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The purpose of the research presented here is to investigate the pathways by which cdk4 and cdk6 stimulate unregulated cell division in cancer cells and the mechanism by which these kinases are differentially activated. Numerous reports have shown that increased expression and protein levels of cdk4 and cyclin D proteins are found in breast cancer, but cdk6 is preferentially activated in oral epithelial cancers. Results summarized here recapitulate these differences in cell culture systems, suggest novel mechanisms of regulation of cdk4 and cdk6 and further support functional differences between these two kinases. A novel method for identifying proteins that differentially interact with the two kinases is also described and studies on several new interactors provide insight into specific potential regulators and substrates of these cdks.

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5. INTRODUCTION

Cancer is characterized in part by a loss of cellular growth control. An important regulator of cellular growth is the retinoblastoma protein, which acts as a negative regulator of cellular proliferation. This protein has been found to be directly inactivated in several tumor types and the vast majority of human tumors contain mutations, amplifications or deletions in a variety of genes referred to as the "pRb pathway" that result in inactivation of pRb (Weinberg, 1995; Sherr, 1996). Deregulation of the pRb pathway can result from mutations of both positive and negative regulators of pRb. For example, in breast cancers cyclin D1 is often amplified. Cyclin D1 activates cyclin dependent kinase 4 (cdk4) and cdk6, both of which phosphorylate pRb to inactivate its cell cycle inhibitory function and contribute to progression toward S-phase. In addition, inhibitors of kinase function are often deleted in cancers (Tiemann et al., 1997).

The cyclin D-dependent kinases play a pivotal role linking growth regulatory signals to cell division. Thus, the activity of these kinases is very tightly controlled through periodic synthesis and destruction of the cyclin subunits, by phosphorylation and dephosphorylation of the kinase subunit, and through complex formation with two families of cyclin-dependent kinase inhibitors (CKIs) (Sherr, 1994; Morgan, 1995; Sherr and Roberts, 1995).

In addition to differences in the particular element of the pRb pathway that is altered in tumors, different cancer types have been found to preferentially activate either cdk4 or cdk6. The purpose of the research presented here is to investigate the pathways by which cdk4 and cdk6 stimulate unregulated cell division and the mechanism by which these kinases are differentially activated. Numerous reports have shown that increased expression and protein levels of cdk4 and cyclin D proteins are found in breast cancer (Tiemann, et al., 1997), but cdk6 is preferentially activated in oral epithelial cancers (Timmermann, et al., 1997). Results summarized here recapitulate these differences in cell culture systems, suggest novel mechanisms of regulation of cdk4 and cdk6 and further support functional differences between these two kinases (Grossel, et al., 1999a). A novel method for identifying proteins that differentially interact with the two kinases is also described (Grossel, et al., 1999b) and studies on several new interactors provide insight into specific potential regulators and substrates of these cdks.

6. BODY

For the final year of this fellowship, a new SOW was produced that divided work among Dr. Martha Grossel, the original PI, now an Assistant Professor at Connecticut College, and Dr. Kathryn Marsh, a Research Fellow in Dr. Phil Hinds' lab at Harvard Medical School. This SOW extends work begun by Dr. Grossel that resulted in publication of a paper in the Journal of Biological Chemistry demonstrating that cdk6 required nuclear localization to act as an accelerator of G1 phase in transfected cells. This function required an intact cdk6 N-terminus and suggested a novel method of spatial regulation of cdk6 that differs from cdk4. This further

suggests that one reason for selection of activation of a specific cyclin D-dependent kinase in a certain cell type may depend on other elements that allow proper localization and activation of the kinase. A reprint of this work is included and further details will not be discussed here (Grossel et al., 1999a).

To identify proteins that might mediate these spatial differences between cdk4 and cdk6, or that might be substrates for one but not both kinases, Dr. Grossel developed a modified two hybrid system that allowed screening of library plasmids that encode proteins that interact with only one or both of two simultaneously expressed baits. This work is also published (Grossel et al., 1999b) and the details of the system are available in the included reprint.

Using the two-hybrid system, the first elements of both Dr. Grossel's and Dr. Marsh's SOW have been significantly advanced. The initial screen for cdk6 interactors yielded 19 confirmed library clones from 1.2 million screened. Table one below summarizes the number and identity of these interactors.

TABLE 1. Interactors with cdk6 and cdk4 identified in differential two-hybrid

Identity	Function	No. of clones	Cdk6 binding	Cdk4 binding
Cyclin D3	Activator of cdk4 and cdk6	1	+	+
P19INK4D	Inhibitor of cdk6	3	+++	ND
Unknown 1	NA	2	+	ND
Unknown 2	NA	1	+	ND
Lamin B2	Nuclear structure	2	+++	ND
Eya2	Transcription coactivator with role in retinal and limb development	1	++	+
RBCK1/2	Unknown	3	++	+/-
KIF2	Vesicle transport in axonal elongation	6	+++	-

The ability of this system to identify legitimate interactors with cdk6 was confirmed by the independent recovery of cyclin D3 and p19INK4d from the library. Of greatest interest are cDNAs encoding three proteins that have not previously been identified as cdk6 interactors. Each of these has undergone initial analysis and all are presently being studied at Harvard Medical School. The following paragraphs give a brief summary of the potential importance of these interactions and our present understanding of their physiological relevance.

Eya2: This protein is intriguing because of its role in early development and fate determination (Mishima and Tomarev, 1998; Heanue, et al., 1999). The connection to retinal development is

particularly intriguing in light of the pRb pathway's high penetrance in cancers of this tissue. We have produced a flag-tagged version of this protein and have detected its expression in transfected cells, but cannot immunoprecipitate the tagged protein. Confirmation of interaction with cdk6 (or cdk4) in cells awaits the production of an Eya2-specific antiserum. We have produced the Eya2 protein both as a GST fusion and in an in vitro translation system. Preliminary data using labeled Eya2 produced in vitro suggests that Eya2 can bind to GST-fused cdk6, extending the original observation of association of these proteins in yeast cells. Experiments are currently underway to test the possibility that Eya2 is a cdk substrate. Of all the proteins identified in this screen, Eya2 is the best candidate for a "new" substrate of cdks, since it contains 11 potential cdk phosphorylation sites within its 500 amino acids. Phosphorylation of Eya2 by cdk6 or a related G1 cdk would raise an intriguing connection between cell cycle control and regulation of transcription in development.

RBCK1/2: The function of these proteins, produced from splice variants of the same gene, is unknown (Tokunaga et al., 1998a; 1998b). However, the shorter form of the protein contains a potential coiled coil domain and a zinc finger domain most closely related to that in RanBP1, a protein that can interact with exportin or CRM1. The longer form of the protein, RBCK1, also contains a ring finger that can mediate an association with UbcH4, a component of the ubiquitination/degradation machinery (Martinez-Noel, et al., 1999). Thus, one inference that will be tested is that the RBCK proteins may mediate export of associated proteins from the nucleus and perhaps mediate ubiquitination and degradation of transported proteins.

We have confirmed the interaction of cdk6 with RBCK1 and 2 by cotransfection and coimmunoprecipitation in mammalian cells. Interestingly, cdk4 does not interact with RBCK1 and 2 in the same assay, supporting the results from the yeast experiment and suggesting that RBCK may mediate cdk6-specific effects. Immunofluorescence studies of cotransfected cells indicates that cdk6 in cells overexpressing RBCK is localized exclusively in the cytoplasm, as is the RBCK protein itself, further supporting a transport role for RBCK. Given the probable spatial regulation of cdk6 reported earlier (Grossel et al., 1999a), RBCK proteins become interesting candidates for specific mediators of cdk6 localization. This possibility is now being vigorously investigated.

KIF2: KIF2 is a member of the kinesin family of motor proteins that is specifically expressed in developing brain and is involved in neurite outgrowth (Noda, et al., 1995; Takemura, et al., 1996; Debernardi, et al., 1997; Morfini, et al., 1997). This potential cdk6 interactor is of particular interest because it represented 6 of the total of 19 clones, interacts strongly with cdk6 in yeast and shows no affinity for cdk4. Cotransfection of cdk6 and KIF2 in mammalian cells results in a profound decrease in KIF2 levels. Introduction of inactive cdk6 or inhibitors of cdk6 mitigates this reduction, suggesting that cdk6 activity may be required for KIF2 reduction. Further studies of the functional relationship between KIF2 and cdk6 are underway. As with

Eya2, above, this potential interaction raises the intriguing possibility of regulation of differentiative processes by the G1 cell cycle machinery, and this in turn could significantly impact the function of cdk6 as an oncogene.

Different functions of cdk4 and cdk6 in primary astrocytes and breast epithelial cells

Recent studies have shown that cdk4 can immortalize primary murine astrocytes. Using the same system, we have found that cdk6 is relatively poor at this. Interestingly, cdk6 is localized exclusively in the cytoplasm in transduced cells, whereas cdk4 is exclusively nuclear. These results further support the notion that spatial regulation of the cdks is different in different cell types and this regulation may contribute to selection of one or another kinase's activity in tumor cells. We are exploring this using mutant versions of the kinases expressed from retroviruses specifically able to infect the murine cells used. Reagent preparation is complete and studies are now being perfomed in the Grossel lab at Connecticut College to elucidate this differential regulation of the cdks. At Harvard Medical School, similar reagents will be used to infect primary breast epithelial cells with the goal of assessing specificity here, since cdk4 activation appears to be preferred in tumors arising from this tissue.

7. Key Research Accomplishments

- Identification of spatial requirements for cdk6 function and specific ability of cdk6 to drive cells through G1 phase in certain cell types.
- Creation of Differential Two-Hybrid System
- Identification of putative cdk6 interactors with a role in cdk6 localization or that link G1 cdk function to differentiation and tumorigenesis.
- Initial studies in deliniation of specific roles for cdk4 and cdk6 in astrocyte proliferation and function.

8. Reportable Outcomes

- Grossel, M.J., Baker, G.L and Hinds, P.W. (1999). Cdk6 can shorten G1 phase dependent upon the N-terminal INK4C interaction domain. J. Biol. Chem. 274: 29960-29967.

  Grossel, M.J., Wang, H., Gadea, B., Yeung, W. and Hinds, P.W. (1999). A yeast two-hybrid system for selecting differential interactions using multiple baits. Nature Biotech. 17: 1232-1233.

- Development of yeast strain HW18 for differential two hybrid; to be deposited at ATCC
- Appointment of Dr. Grossel as Assistant Professor at Connecticut College

9. Conclusions

This work demonstrates distinct, cell-type specific roles for cdk4 and cdk6 that depend in part on their different spatial regulation within the same cell. These differences in function of members of the pRb pathway are likely to contribute significantly to the role of these kinases as oncogenes in certain tissue types. The preference for one or another of these kinases may depend on the availability of spatial regulators; to that end we have sought to identify proteins involved in this regulation of cdks. Further, some cell type specific functions of cdk4 and cdk6 may reflect different interactions with substrates and these have also been investigated here. The development of a two-hybrid system that allows direct comparison of interactors with two baits has facilitated the aforementioned identification of cdk-specific interactors. Using this system, we have identified at least three very intriguing cdk6 interactors that have great potential in expanding our understanding of the role of cdk6 in development and cancer.

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11. Appendices

Reprint of:

Grossel, M.J., Baker, G.L and Hinds, P.W. (1999). Cdk6 can shorten G1 phase dependent upon the N-terminal INK4C interaction domain. J. Biol. Chem. 274: 29960-29967.

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Meeting Abstracts:

CDK6, BUT NOT CDK4, CAN SHORTEN G1 PHASE DEPENDENT UPON THE $P18^{INK4C}$ INTERACTION DOMAIN. Martha J. Grossel, Gregory L. Baker and Philip W. Hinds, Harvard Medical School, Department of Pathology, Boston, MA 02115. NIH GM5568, Army Breast Cancer DAMD17-97-1-7167

IDENTIFICATION OF CDK6-SPECIFIC INTERACTORS USING A MODIFIED YEAST TWO-HYBRID SCREEN.
Nathan W. Swilling, Martha J. Grossel, Gregory L. Baker, Hongmei Wang and Philip W. Hinds; Harvard Medical School, Department of Pathology, Boston, MA 02115; Cold Spring Harbor Meeting on Cell Cycle Control, 4/2000.

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Martha Grossel
Kathryn Marsh

IN THE LABORATORY

A yeast two-hybrid system for discerning differential interactions using multiple baits

*Martha J. Grossel[1], Hongmei Wang, Bedrick Gadea, Wilbert Yeung, and Philip W. Hinds**

The yeast two-hybrid system is a powerful genetic technique designed to identify novel protein–protein interactions that were previously limited to detection by biochemical studies. The original system[1] has been manipulated and improved upon by interchanging DNA-binding domains[2], by adding multiple reporter constructs[2–4], and by using a reverse system to study disruption of protein interactions[4,5].

All two-hybrid screening systems rely on the fact that transcriptional activation and DNA-binding domains of transcription factors are modular in nature[6,7]. In these systems, the coding sequence for the DNA-binding domain of a transcription factor such as Gal4 or LexA is fused to the cDNA of a protein of interest, termed the "bait". The fusion protein thus encoded tethers the bait to the promoter region of a reporter gene. A second fusion of a cDNA library with the coding sequence of a transcriptional activation domain is termed the "prey". Functional reconstitution of transcription factor activity occurs upon association of the bait and prey protein domains. This interaction is then detected by expression of reporter genes that are dependent upon the bait's DNA-binding domain.

Thus, the two-hybrid system is ideal for screening libraries for novel protein–protein interactions, and is also a powerful tool to isolate factors that disrupt or promote protein interactions. Its advantage is that an interaction of the bait with an unknown prey leads to direct identification of the cDNA that encodes the interacting protein, and its use in high-throughput screening applications allows the rapid and efficient screening of large numbers of potential interacting cDNAs or peptides.

Many proteins screened in the two-hybrid system are members of larger protein families. It is often of interest to screen closely related family members for their ability to interact with binding partners identified in the original screen. A system that would permit the screening of two homologous bait proteins in one yeast would allow direct comparisons of binding partners within a family of proteins. This system would be more efficient and would permit the simultaneous identification of proteins that interacted with either one of the two bait proteins and, at the same time, proteins that interacted with both of the related baits. This system could also be used to discern similarities and differences in binding partners of wild-type and mutant forms of a protein.

We describe a "differential" two-hybrid yeast system that can screen for interactions between prey proteins and two different bait proteins through the activation of bait-specific reporters. It allows the identification of proteins that interact differentially with one bait tethered to the Gal4 DNA-binding domain and another bait tethered to the LexA DNA-binding domain. The system also allows the identification of elements that specifically disrupt protein–protein interactions of either bait protein or both.

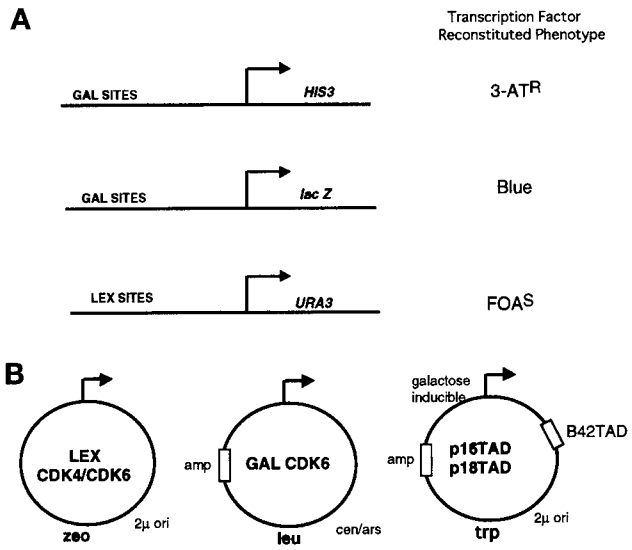

Figure 1. Differential yeast strain HW18. (A) Eight LEXA binding sites were introduced into the *SPO13* promoter of *URA3* to create the differential strain HW18. The interaction of a bait fusion with a prey fusion reconstitutes the transcription factor resulting in the indicated phenotype. (B) Plasmids pLexcdk4, pLexcdk6, pGalcdk6, and p16TAD or p18TAD were constructed and expressed as described.

Differential yeast strain HW18

A yeast strain was constructed in which the transcription of two reporter genes, *HIS3* and *lacZ*, is dependent upon GAL4 binding sites in their promoters, and transcription of the third reporter gene, *URA3*, is dependent on LEXA binding sites in the promoter region (see Fig. 1A). This yeast strain allows screening of two different proteins or "baits" fused to two different DNA-binding domains, each bait activating heterologous (GAL4-driven or LEXA-driven) reporter genes. When the GAL4 DNA-binding domain fusion protein (GAL DBD) interacts with a cDNA library-encoded protein fused to a *trans*-activation domain (TAD), this protein–protein interaction activates the two GAL4-dependent reporter genes. A second bait fused to the LexA DNA-binding domain (LEX DBD) activates the LEXA-dependent reporter, *URA3*, when it interacts with a prey–TAD fusion protein. Thus, a two-hybrid screen can be performed with two different baits at the same time, with each bait activating individual reporter genes.

The strain described, HW18, is derived from MaV52 (ref. 4) by inserting eight LEXA binding sites into the highly repressive *SPO13* promoter driving the *URA3* open reading frame. DNA gel analysis of PCR reactions that specifically amplified the *URA3* promoter region from the yeast chromosome confirmed that yeast strain HW18 contains eight LEXA binding sites (data not shown). Importantly, this *SPO13* promoter of *URA3* confers a Ura⁻ phenotype in the absence of a LexA-based transcription factor.

Plasmids that encoded fusions of cdk6 and cdk4 cDNAs with genes for Gal4 or LexA DNA-binding domains were introduced into the HW18 yeast strain (see Fig. 1B). The pGalcdk6 plasmid contains the Gal4 DBD in reading frame with the cDNA of cdk6 and the *leu2* marker for auxotrophic selection. pLexcdk4 contains the LexA DBD in frame with the cdk4 cDNA and contains the *zeocin* (*zeo*) resistance selectable marker. In addition, plasmid pLexcdk6 contains the cdk6 cDNA in frame with the LexA DBD. The third plasmid in these studies contains either cDNAs of known cdk4 and cdk6 interacting proteins in frame with B42 TAD and the *trp1* marker (to characterize the system) or a cDNA library fused to the GAL4 TAD to screen for protein

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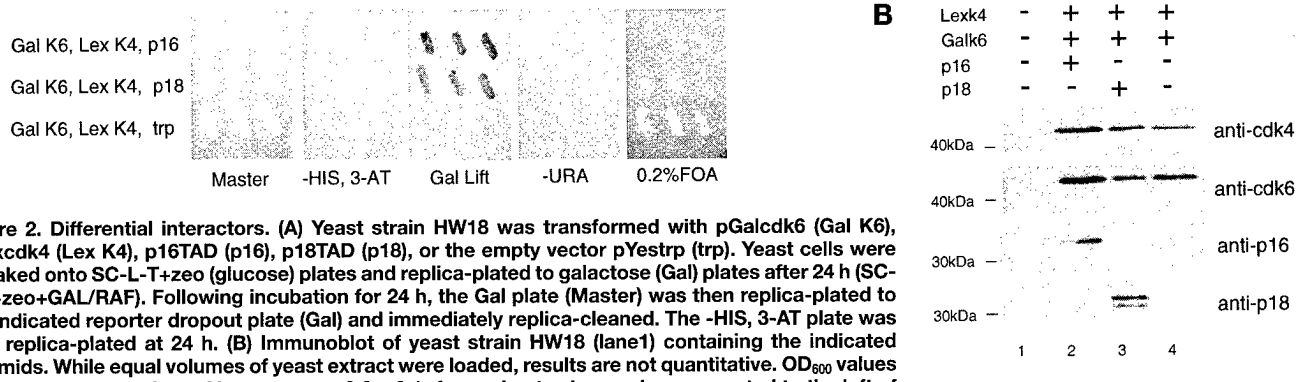

Figure 2. Differential interactors. (A) Yeast strain HW18 was transformed with pGalcdk6 (Gal K6), pLexcdk4 (Lex K4), p16TAD (p16), p18TAD (p18), or the empty vector pYestrp (trp). Yeast cells were streaked onto SC-L-T+zeo (glucose) plates and replica-plated to galactose (Gal) plates after 24 h (SC-L-T+zeo+GAL/RAF). Following incubation for 24 h, the Gal plate (Master) was then replica-plated to the indicated reporter dropout plate (Gal) and immediately replica-cleaned. The -HIS, 3-AT plate was also replica-plated at 24 h. (B) Immunoblot of yeast strain HW18 (lane1) containing the indicated plasmids. While equal volumes of yeast extract were loaded, results are not quantitative. OD_{600} values of yeast extracts at time of harvest were 0.6 ± 0.1. Approximate size markers are noted to the left of the panels. A single blot was repeatedly stripped and reprobed using the indicated antibodies.

interactions. Yeast strain HW18 was transformed with these *zeo, leu2,* and *trp1* plasmids and selected on SC-L (Leu), -T(Trp), +zeo (300 μg/ml) media and tested for activation of the three reporter genes.

Discriminating interactions of a prey with two different baits

This system was designed to differentiate the interaction of a prey protein with two different bait proteins, thus allowing the identification of a cDNA that encodes a protein that interacts with either one or both of the bait fusions. To test its utility for such applications, the interaction of two different preys was tested for interaction with Galcdk6 and Lexcdk4.

As shown in Figure 2A, while p16 interacts with both Galcdk6 and Lexcdk4, the p18 protein preferentially interacts with cdk6. This differential interaction is easily revealed by the phenotype of the yeast containing the interactors. Upon interaction with Galcdk6, both p16 and p18 activated the *lacZ* gene and the *HIS3* gene as evidenced by blue color formation and growth on 20 mM 3-AT plates. Importantly, the activation of both reporters was dependent upon protein–protein interactions, as evidenced by a lack of activation in the empty plasmid control strain, which contains the B42 TAD but no cDNA fusion (see Fig. 2A). The p16 protein also interacted with Lexcdk4 to activate the *URA3* gene as demonstrated by growth on uracil dropout plates and by lack of growth on 0.2% FOA plates. When the p18 fusion was transformed into yeast containing Galcdk6 and Lexcdk4, the yeast scored positive for lacZ production and grew on 20 mM 3-AT media. These Gal4-driven reporters indicate that the cdk6/p18 interaction is clearly able to reconstitute transcription factor activity, although the p18 interaction with Galcdk6 may be slightly weaker than the p16 interaction with Galcdk6. Most importantly, p18TAD could not productively interact with Lexcdk4 in the same strain, as evidenced by lack of growth on Ura dropout plates (-URA) and by resistance to 0.2% FOA.

Immunoblots in Figure 2B demonstrate that Lexcdk4 and p18 protein are present in this strain, confirming that the p18 protein was produced, yet was unable to interact with Lexcdk4. This is consistent with a previous report indicating that p18 binds cdk6 but not cdk4 in a two-hybrid system[8]. These findings indicate that this system is suitable for detecting differential interaction of a bait protein (p18) with two different prey proteins (cdk4, cdk6).

Library screening

A partial screen of a cDNA library fused to the GAL4 TAD (Gibco/BRL) resulted in clones that interacted with the Galcdk6 bait but not the Lexcdk4 bait. The same screen also isolated cDNAs that activate both LEXA- and GAL4-dependent reporters as well as cDNAs that activated GAL4-dependent reporters weakly and the LEXA-dependent reporter strongly. Interestingly, one cdk6-specific interactor isolated is homologous to a rat kinase-binding protein. The identification of a kinase-binding protein that differentiates between the highly homologous kinases used as baits, cdk4 and cdk6, indicates that the system can isolate proteins or peptides that bind specifically to one of two closely related baits.

Conclusions

The combination of differently activated reporters allows the study of multiple protein–protein interactions in one strain of yeast and in one screening[9–11]. The system described here was designed to allow screening of cDNA libraries with multiple baits. When used in differential screens, the two Gal4-driven reporters and the single LexA-driven reporter optimize the identification of binding partners for the Gal4 DBD fusion protein and selection for or against the LexA DBD fusion protein. The ability to screen two proteins at once also decreases the labor involved in screening and allows the identification of proteins that bind to either or both bait proteins. This system also eliminates the need for mating two yeast strains to discern interactions with additional proteins[12].

The differential two-hybrid yeast has important applications in mutagenesis studies. For example, the products of a random mutagenesis of a protein can be screened for the persistence of interaction with one binding partner and the simultaneous disruption of a second binding partner. This would allow identification of a mutant protein that specifically disrupts binding of discrete partners while preserving other interactions, thus selecting against mutations that cause global conformational disruptions of the mutagenized protein. Thus, the system can now be enhanced by examining multiple protein interactions or disruptions in one yeast strain in one screen.

Finally, this system should provide a powerful tool for studying the promotion or disruption of protein–protein interactions using peptide or small-molecule libraries. It may be possible to identify compounds that affect the interaction of a (disease-related) mutant form of a protein with a binding partner, but not a wild-type form of the protein with the same binding partner. We believe this system is well suited for discovery of specific interactors with individual members of families of related proteins as well as for high-throughput studies of factors affecting protein–protein associations.

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cdk6 Can Shorten G_1 Phase Dependent upon the N-terminal INK4 Interaction Domain*

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Deregulated activity of cdk4 or cdk6 can lead to inappropriate cellular proliferation and tumorigenesis accompanied by unchecked inactivation of the retinoblastoma tumor suppressor protein. Certain tumor types preferentially activate either cdk4 or cdk6, suggesting that these kinases may not be equivalently oncogenic in all cell types. Although it is clear that cdk4 can act as an oncogene at least in part by evading inhibition by $p16^{INK4a}$, the role of cdk6 in tumorigenesis is less well understood. To investigate the consequences of aberrant expression of cdk6, the requirements for proliferation caused by cdk6 overexpression were studied. cdk6-transfected U2OS cells displayed an accelerated progression through G_1 phase that was dependent on kinase activity and that did not correlate with p27 binding. Furthermore, a mutation that prevents cdk6 interaction with INK4 proteins (cdk6R31C) was found to inactivate the proliferative effect of cdk6 and increase cytoplasmic localization, despite the fact that this mutant could phosphorylate the retinoblastoma protein *in vitro*. Together, these data suggest a role for the cdk6 INK4 interaction domain in the generation of functional, nuclear cdk6 complexes and demonstrate the importance of elevated cdk6 kinase activity in G_1 acceleration.

In mammalian cells, the regulation of cell division is tightly controlled through a series of checkpoints within the cell cycle, including the restriction point in late G_1 phase, a checkpoint that determines commitment to DNA replication. The restriction point may be viewed as the culmination of activation of G_1 cyclin-dependent kinases, enzymes that govern cell cycle progression through phosphorylation of key regulatory substrates. Specifically, the cyclin D proteins and their associated kinases, cyclin-dependent kinase (cdk)[1] 4 and cdk6, function early in G_1 phase of the cell cycle to link growth regulatory signals to the control of cell division. Both cdk4 and cdk6 can be activated by all three D-type cyclins (cyclins D1, D2, and D3) and are thought to function as positive effectors of G_1 progression (1–4). Activation of cdk4 and cdk6 allows progression from G_1 phase to the start of DNA synthesis in normal eukaryotic cells by phosphorylating and inactivating the retinoblastoma protein (pRb). This initial modification of pRb by cdk4/cdk6-dependent phosphorylation may be followed by further phosphorylation by cyclin E/cdk2 complexes and ultimately relieves repression of E2F-dependent promoters, allowing the transcription of S phase genes and the onset of DNA replication (for review, see Ref. 5).

Because the cyclin D-dependent kinases play a pivotal role linking growth regulatory signals to cell division, activity of these kinases is very tightly controlled. Kinase activity is regulated by the periodic synthesis and destruction of the cyclin subunits, by phosphorylation and dephosphorylation of the kinase subunit, and through complex formation with two families of cyclin-dependent kinase inhibitors (CKIs) (6). The CIP/KIP family of inhibitors includes p21, p27, and p57, which associate with several different cyclin/cdk complexes (7–11). These proteins may act as stimulators of cdk activity as well as inhibitors because p21 has been shown to both activate and inactivate cyclin/cdk complexes, perhaps dependent on stoichiometry (12, 13). Indeed, the ability of p21 and p27 to stabilize D cyclin/cdk4 (6) complexes may be required for the proper formation of these complexes (14). In contrast to the CIP/KIP family, the INK family of CKIs ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$) specifically inhibits the activities of cdk4 and cdk6 by binding directly to the kinase subunit, disallowing association with the activating cyclin D subunit (Refs. 15 and 16; for review, see Ref. 17).

Aberrant cell proliferation and tumorigenesis can result from deregulated activity of cdk4 and/or cdk6 with subsequent, inappropriate inactivation of pRb in several tissue types. This increased kinase activity can result from overexpression of the regulatory subunit, cyclin D1, and also from amplification of the kinase-encoding gene. In addition, deletion or inactivation of the gene encoding $p16^{INK4a}$ frequently leads to dysregulated cdk4/cdk6 activity in human tumors, as do mutations in cdk4 that prevent its association with $p16^{INK4a}$ (18–25, 29). In most cases, tumors containing hyperactivated cdk4 or cdk6 retain intact *RB* alleles, suggesting that such kinase activations render pRb unable to control proliferation. These findings indicate that deregulated cdk4 and cdk6 activity can substitute for *RB* mutations and define the "pRb pathway" of genetic events that have the identical phenotypic consequence of pRb inactivation and inappropriate proliferation. A further oncogenic consequence of excess cyclin D/cdk4(6) expression is sequestration of p21 and p27 with consequent elevated activity of cyclin E/cdk2, a function that may be an extension of a physiological role of cyclin D/cdk4 (6) complexes (14, 26–28).

To better understand the consequences of dysregulated cdk6 expression as is seen in some tumor types (30, 31), we began by studying the requirements for proliferation caused by ectopic cdk6 expression. cdk6-transfected cells demonstrated accelerated transit through the G_1 phase of the cell cycle. This effect of cdk6 on cell cycle progression was dependent on the INK4

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[1] The abbreviations used are: cdk, cyclin-dependent kinase; Rb, retinoblastoma; pRb, Rb protein; HA, hemagglutinin; BrdUrd, 5-bromo-2′-deoxyuridine; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate.

binding domain, because a cdk6 mutant (R31C) unable to associate with INK4 proteins did not show G_1 acceleration. In addition, this mutant protein failed to accumulate in the nucleus, suggesting that nuclear localization and function of cdk6 is dependent on the INK4 interaction domain. Furthermore, catalytic activity of cdk6 was required for G_1 acceleration in this assay, because a catalytically inactive NFG mutant slowed S phase entry rather than accelerated it, despite an ability to form complexes with cyclin D1 and p27. Thus, cdk6 activity is limiting for G_1-to-S phase progression, even in tumor cells such as U2OS, which lacks $p16^{INK4a}$, strongly supporting a role for cdk6-specific phosphorylation events in G_1 progression.

EXPERIMENTAL PROCEDURES

Expression Vectors, Transfection Procedures, and Cell Lines—The kinase expression plasmids pCMVcdk6 and HA-tagged cdk6, pCMVcdk6HA, pCMVcdk6NFG, the vector pCMVneobam, and the CD20-encoding plasmid pCMVCD20 were kindly provided by Dr. Sander van den Heuvel (32). pCMVBamNeo, the vector containing the cDNAs, has been described previously (33). U2OS cells were transiently transfected with 15 μg of kinase-expressing plasmid plus 5 μg of pCMVCD20 (where appropriate) and sheared herring sperm DNA to a total of 30 μg by calcium phosphate precipitation essentially as described by Chen and Okayama (34). DNA precipitates remained on the cell monolayer for 17 h, and cells were harvested 24 h after removal of DNA precipitates unless otherwise noted. U2OS cells were maintained in 10% fetal calf serum at 5% CO_2.

Analysis of Cell Cycle Distribution—For fluorescence-activated cell sorting (FACS) experiments, transfected U2OS cells were harvested in PBS with 0.1% EDTA at 24 h after removal of DNA precipitates. Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated antibody to human CD20 (Pharmingen) and ethanol fixed and stained with propidium iodide for DNA content. Cell cycle distribution was analyzed by flow cytometry of CD20-positive (FITC-positive) cells using a Coulter cytometer and Multicycle DNA analysis. In FACS studies of nocodazole-treated cells, nocodazole was added at 24 h after removal of DNA precipitates to a final concentration of 100 ng/ml for 18 h. In FACS studies following mitotic shake, mitotic fractions were harvested by gentle pipetting followed by centrifugation at 1000 rpm at 25 °C for 5 min. Cells were washed three times with media to remove nocodazole and replated in 10% fetal calf serum media. Cells were harvested at time points indicated and prepared for FACS as described above.

BrdUrd and Immunofluorescence—In 5-bromo-2′-deoxyuridine (BrdUrd) incorporation experiments nocodazole was added to 100 ng/ml approximately 5 h after removal of DNA precipitates and remained on cells 18 h. BrdUrd was added to a final concentration of 10 μM at the time points indicated. Coverslips from BrdUrd experiments were fixed in 70% ethanol, 50 mM glycine, pH 2.0, and incubated with BrdUrd monoclonal antibody (Roche Molecular Biochemicals) and polyclonal peptide antibodies to cdk6 (Santa Cruz Biotechnology, C-21) for 60 min at 37 °C. Secondary antibodies rhodamine-conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA) and fluorescein-conjugated anti-mouse (Roche Molecular Biochemicals) were incubated 30 min at 37 °C. Coverslips were mounted in Fluoromount. For time courses, mitotic shake was performed as described above, and coverslips were fixed at the indicated time points. For immunofluorescence without BrdUrd, coverslips were stained in methanol followed by acetone. Immunofluorescence was performed with antibodies indicated above, as well as cdk6 polyclonal sera of Meyerson (4) and cdk6 monoclonal sera Ab-3 (Neomarkers, Fremont, CA) for 60 min at 37 °C. Secondary antibody staining was performed as described above or with fluorescein-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch). In relevant cases, cells were counterstained by Hoechst stain. All photography was performed on a Leica microscope with Sony digital imaging.

Biochemical Assays—For immunoblot and immunoprecipitation experiments, 1.25×10^6 U2OS cells were transfected as described above, harvested 24 h after removal of DNA precipitates, washed twice with phosphate-buffered saline and harvested in E1A lysis buffer (250 mM NaCl, 50 mM Hepes, pH 7.0, 5 mM EDTA, 0.1% Nonidet P-40). Extracts were incubated for 20 min on ice with mixing and clarified by centrifugation for 20 min at 4 °C. Proteins were separated on polyacrylamide denaturing gels, transferred to supported nitrocellulose (Life Technologies, Inc.) and blotted using antisera as noted.

Immunoprecipitations were performed with 2 μg of polyclonal cdk6 antisera C-21. Transfected U2OS cells were lysed in E1A lysis buffer as described above, and 200 μg (cyclin D1) or 400 μg (p18, p27) of extract was immunoprecipitated for 60 min at 4 °C with mixing. 35 μl of swollen protein A-Sepharose beads were added for an additional 30 min, washed four times with 1 ml of E1A lysis buffer, and separated on denaturing acrylamide gels. Antibodies used were p18 polyclonal N-20 antibody (1:3000) (Santa Cruz), p27 monoclonal antibody (1:2500) (Transduction Laboratories), and cyclin D1 monoclonal DCS-6 antibody (1:200) (Neomarkers).

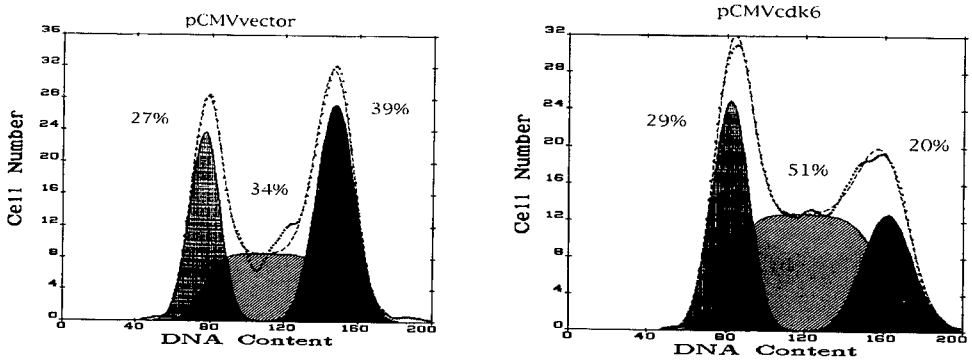
For kinase assays, SAOS-2 cells at 80% confluency were transfected with 10 μg of pCMVD1 and 10 μg of pCMVcdk6HA or cdk6mutantHA in the pCMV vector by calcium phosphate as described above. DNA precipitates remained on cells for 10 h and were harvested 36 h after removal of DNA precipitates. Cells were harvested in D-IP kinase buffer (50 mM Hepes, pH 7.5; 150 mM NaCl; 1 mM EDTA; 2.5 mM EGTA; 0.1% Tween 20; 10% glycerol with the protease inhibitors aprotinin, leupeptin, and Pefablock and phosphatase inhibitors sodium orthovanadate (100 μM), sodium flouride (10 mM), and betaglycerophosphate (10 mM)) and incubated on ice for 20 min with gentle mixing. Lysates were clarified by centrifugation at 4 °C for 10 min. 100 μl of 12CA5 antibody was preincubated with 30 μl of swollen protein A-Sepharose beads for at least 1 h at 4 °C with mixing. 100 μg of cell lysate was added for an additional 1 h at 4 °C with mixing. Beads were washed three times with D-IP buffer and three times with kinase reaction buffer (250 mM Hepes, pH 7.2, 50 mM $MgCl_2$, 25 mM $MnCl_2$, 1 mM dithiothreitol). Kinase reactions were performed with HA-immunoprecipitated extracts at 37 °C for 30 min in kinase reaction buffer with 100 μM ATP, 10 μCi of [γ-^{32}P]ATP and 0.5 μg of C-terminal GST-Rb (amino acids 769–921) (Santa-Cruz Biotechnology) as substrate. Reactions were stopped by addition of protein sample buffer with 10% betamercaptoethanol and placed on ice. Samples were boiled and separated on 12.5% denaturing acrylamide gel, Coomassie Brilliant Blue-stained to ensure equal loading and addition of Rb substrate, and exposed to film overnight.

RESULTS

cdk6 Causes Increased S Phase of Transfected Cell Populations—The effect of ectopic expression of cdk6 on cell cycle progression was determined by FACS studies in transfected U2OS cells. These human osteosarcoma cells produce wild-type pRb but lack $p16^{INK4a}$, a defect that is thought to allow constitutive phosphorylation and inactivation of pRb. In light of this, it was surprising to observe that cells transfected with plasmid encoding cdk6 consistently showed a higher percentage of S phase cells than did vector-transfected cells in the same experiment (Fig. 1). To confirm that this increase in DNA content measured by FACS truly reflected an increase in S phase cells, transfected cell cultures were also analyzed using BrdUrd incorporation as a measure of S phase. U2OS cells transfected with pCMVcdk6 or pCMVvector were pulsed with BrdUrd, fixed, and subjected to indirect immunofluorescence using both anti-BrdUrd and anti-cdk6 antibodies. Transfected cells were identified as those that demonstrated intense fluorescence with anti-cdk6 antibody and were scored as either BrdUrd-positive or BrdUrd-negative. The results of at least two independent transfections (≥700 kinase-positive cells counted) demonstrated that 34% of vector-transfected cells were BrdUrd-positive, whereas in the same experiment, 50% of cdk6-transfected cells were BrdUrd-positive (Fig. 1*B*). These data closely match the results of FACS analysis shown in Fig. 1*A* and indicate that cells transfected with cdk6 showed a statistically significant ($p < 0.05$) increase in the percentage of S phase population as compared with vector-transfected cells.

cdk6 Shortens the G_1 Interval of Transfected U2OS Cells—The observed increase in S phase cells conferred by cdk6 could result from either an S phase cell cycle block or from decreased transit time through G_1 or G_2/M. To distinguish between these possibilities, transfected cells were treated with the mitotic inhibitor nocodazole. In the presence of nocodazole, an S phase delay would reduce the number of cells able to enter G_2/M. However, if the increased S phase population was due to a shortening of G_1 phase (or G_2/M phase) cells would arrest in mitosis under nocodazole treatment. At 24 h after removal of

A



B

plasmid transfected	cells counted	Kinase + BrdUrd +	Kinase + BrdUrd -
pCMVvector	732	34%	66%
pCMVCDK6	902	50%	50%

FIG. 1. **DNA profiles and BrdUrd incorporation of vector and cdk6-transfected cells.** *A,* U2OS cells were transfected with indicated plasmids and harvested for FACS analysis. Cells were incubated with FITC-conjugated CD20 antibody to distinguish transfected populations, fixed, and stained with propidium iodine for DNA content. At least 1800 CD20-positive events were counted per histogram. Results are representative of those found in at least three independent experiments. *B,* BrdUrd incorporation for vector- and cdk6-transfected cells. Values represent the average of at least two independent experiments for which a total of at least 700 events were counted.

DNA precipitates, parallel sets of transfected U2OS cells were either harvested or treated with nocodazole for 18 h. A p16 control for nocodazole arrest demonstrated that p16 transfected cells maintained a G_1 phase peak as expected with a G_1-arresting inhibitor (Fig. 2*A*). In the same experiment, cdk6-transfected cells accumulated in mitosis in the presence of nocodazole, indicating that the S phase increase seen by FACS and BrdUrd incorporation studies (Fig. 1) was not due to a profound S phase delay but was more likely a result of a decreased G_1 or G_2/M transit time.

To determine whether ectopic cdk6 expression decreased G_1 phase transit time, FACS analysis was performed on synchronized cell populations. Transfected U2OS cells were treated with nocodazole for 18 h followed by shaking and replating in nocodazole-free medium. The cells were harvested at 4 and 8 h after mitotic shake, and DNA profiles of CD20-positive cells were obtained by FACS as shown in Fig. 2*B*. At 4 h after mitotic shake, cdk6-transfected cells showed a synchronized DNA profile indistinguishable from that of vector transfected cells. Interestingly, at 8 h post-mitotic shake, cdk6-transfected cells showed a shift toward S phase (increased DNA content) as compared with vector-transfected cells. Together, these experiments indicate that cdk6-transfected U2OS cells pass through G_1 phase faster than vector-transfected cells, demonstrating a cdk6-dependent acceleration of G_1 transit in U2OS cells. Also shown in Fig. 2*B* are DNA profiles of a mutant form of cdk6, cdk6R31C. cdk6R31C contains a mutation of the arginine residue corresponding to Arg-24 in cdk4. Mutation of this residue (Arg-24 to Cys) in cdk4 was identified in a human melanoma and prevents binding to the kinase inhibitor, p16 (24). Interestingly, cdk6R31C did not demonstrate the shift toward S phase seen with the wild-type cdk6, suggesting a role for the INK4 binding domain in the G_1 acceleration function of cdk6. The lack of G_1 acceleration exhibited by cdk6R31C could be due to an unexpected loss of catalytic activity or to the disruption of another property of cdk6 required for this G_1 acceleration function in U2OS cells.

Biochemical Characterization of cdk6 Mutants—The acceleration of G_1 phase caused by ectopic expression of cdk6 could be the result of direct catalytic activity of the introduced kinase subunit phosphorylating substrates such as pRb to shorten G_1 phase. Alternatively, excess kinase subunits could titrate inhibitory proteins to allow activation of other cdks and concomitant cell cycle advance. Titration of inhibitory proteins has been observed to occur upon introduction of both functional and nonfunctional kinases in another system, apparently through titration of p21 (28), and cyclin D/cdk4(6) complexes have been suggested to sequester p27 in the absence of anti-mitogenic signals (26, 27). In an effort to determine the properties of cdk6 required to accelerate G_1 progression in U2OS cells, a series of cdk6 mutants compromised in their ability to bind to INK4 protein (cdk6R31C), hydrolyze ATP (cdk6NFG) (32), or both (cdk6R31CNFG) were used in cell cycle analyses. The biochemical characterization of these mutant proteins is presented in Fig. 3. All cdk6 mutants consistently showed approximately equal protein levels in transfected U2OS lysates (Fig. 3*A*). Consistent with the predicted result, we found that the R31C mutation prevented interaction with $p16^{INK4a}$ (data not shown) and $p18^{INK4c}$ in transfected U2OS lysates (Fig. 3*B*), as has also been observed in breast cancer cell lines (35). The $p18^{INK4c}$ interaction is particularly relevant to U2OS cells because these cells lack $p16^{INK4a}$ yet express detectable levels of $p18^{INK4c}$ bound to cdk6 (data not shown). Disruption of INK4 binding occurred whether the mutation was present alone (cdk6R31C) or in combination (cdk6R31CNFG) with the catalytically inactive mutation (Fig. 3*B*). Importantly, the R31C mutation does not disrupt the ability of cdk6 to bind cyclin D1 or p27 in immunoprecipitations of transfected U2OS extracts (Fig. 3, *C* and *D*). In these experiments, immunoblots were stripped and reprobed with anti-cdk6 antibody to ensure that equivalent

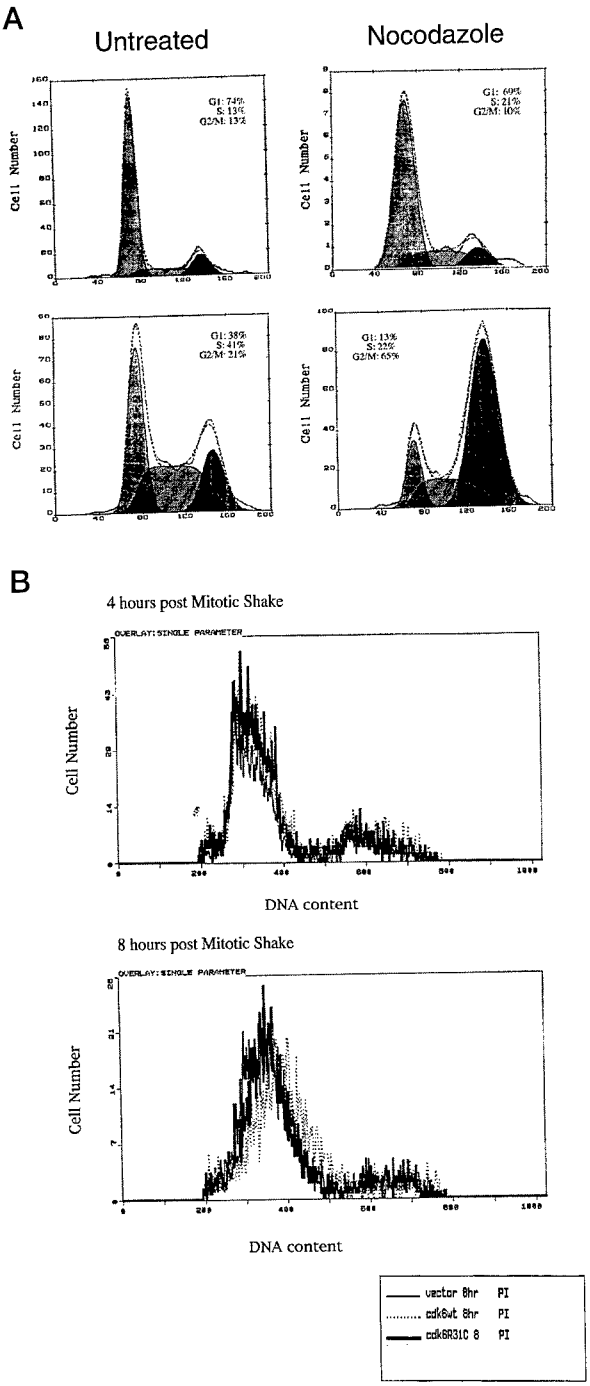


FIG. 2. **Cell cycle profiles of transfected cells.** Cells were incubated with FITC-conjugated CD20 antibody to distinguish transfected populations, fixed, and stained with propidium iodine for DNA content. *A,* FACS profiles of p16- and cdk6-transfected U2OS cells either untreated or nocodazole-treated, as indicated. *B,* DNA profiles of nocodozole synchronized cells transfected with vector (*solid line*), cdk6 (*dotted line*), or cdk6R31C (*boldface line*) and harvested at 4 and 8 h following mitotic shake and replating.

levels of cdk6 protein were compared in binding studies, and control immunoblots of the lysates demonstrated that cyclin D1 or p27 levels were equivalent in extract of transfected cells (data not shown). Thus, the cdk6R31C mutation that corresponds to the tumor-derived cdk4R24C mutation (24) specifically disrupts cdk6 binding to INK4 proteins without altering interaction with other known cdk6 partners.

To ensure that the R31C mutant form of cdk6 retained catalytic activity, the cdk6 mutants were also examined for kinase activity in transfected SAOS-2 cells (used in these assays because they contain low-levels of endogenous cyclin D1 and cdk6 activity). As shown in Fig. 3*E,* when co-transfected with cyclin D1 and immunoprecipitated with antibody to the HA tag, HAcdk6 phosphorylated the C-terminal GST-Rb substrate. Conversely, cdk6NFG containing the kinase-inactivating mutation showed no kinase activity above vector-transfected background. Significantly, cdk6R31C-transfected extracts reproducibly showed *in vitro* kinase activity greater than that observed with wild-type cdk6 extracts, as expected for a mutant that can evade the $p16^{INK4a}$ present at high level in SAOS-2 cells. Anti-HA immunoblots of these extracts confirmed that the level of cdk6R31C was at or below the level of wild-type cdk6 protein (not shown). Interestingly, the double mutant cdk6R31CNFG had a slightly elevated activity relative to the inactive NFG mutant. This result suggests that a low level cdk6NFG activity is unmasked in cdk6R31CNFG by the disruption of INK binding.

Thus, the cdk6R31C mutation disrupts INK4 protein binding but did not disrupt intrinsic catalytic activity of this cdk6 protein, similar to studies demonstrating retention of kinase activity by the $p16^{INK4a}$ binding-defective mutant of cdk4, cdk4R24C (24). Importantly, these binding studies and kinase assays also indicate that these mutations are not causing gross structural alterations in the cdk6 protein. These reagents are thus ideal for assessing the potential roles of catalytic activity and INK4 titration in the cdk6-mediated acceleration of G_1 phase in transfected U2OS cells.

cdk6 Mutants Do Not Accelerate G_1 Phase—The cdk6 mutants described above were used to further examine cdk6 function in G_1 acceleration of U2OS cells. To test the ability of mutant forms of cdk6 to decrease G_1 transit time, BrdUrd incorporation was used to measure S phase entry by the cdk6 mutants. In these experiments BrdUrd was added to transfected U2OS cells 4 h after mitotic shake and BrdUrd incorporation was measured at 5, 7, and 10 h after mitotic shake. The results of these experiments in which cumulative BrdUrd incorporation was measured are presented in Fig. 4. These BrdUrd studies indicated that the kinase inactive mutant, cdk6NFG, showed a slower entry into S phase as compared with the vector-transfected control. In fact, cdk6NFG showed a greatly decreased percent of cells in S phase at all time points measured (27% at 10 h). FACS analysis after nocodazole treatment suggested that this decrease in S phase entry was due to a G_1 delay because a significant G_1 fraction persisted in nocodazole-arrested cells (not shown). A similar delay to S phase entry has been observed with cdk4NFG (36). Thus, catalytic activity in addition to inhibitor titration appears to be required for the G_1 acceleration observed with wild-type cdk6 because cdk6NFG was incapable of increasing the S phase fraction of transfected cells but is fully capable of inhibitor interaction.

Because the result above suggests that kinase activity intrinsic to cdk6 is key to accelerating G_1 phase in transfected U2OS cells, the cdk6R31C mutant initially was expected to give an increase in S phase cells equal to or greater than that conferred by wild-type cdk6, given the ability of cdk6R31C to phosphorylate pRb and avoid interaction with INK4 proteins. However, consistent with results in Fig. 2*B*, cdk6R31C did not show the G_1 acceleration typical of wild-type cdk6, as might be expected if $p18^{INK4c}$ acts to limit cdk6 activity in these cells. In fact, both cdk6R31C (41%) and cdk6R31CNFG (38%) show significantly fewer cells in S phase than did cdk6wt (60%) at 10 h after release from mitotic block. The values for the cdk6R31C and cdk6R31CNFG mutants were similar to that of the vector-transfected control (39%). Thus, intrinsic kinase activity appears to be necessary but not sufficient for the increase

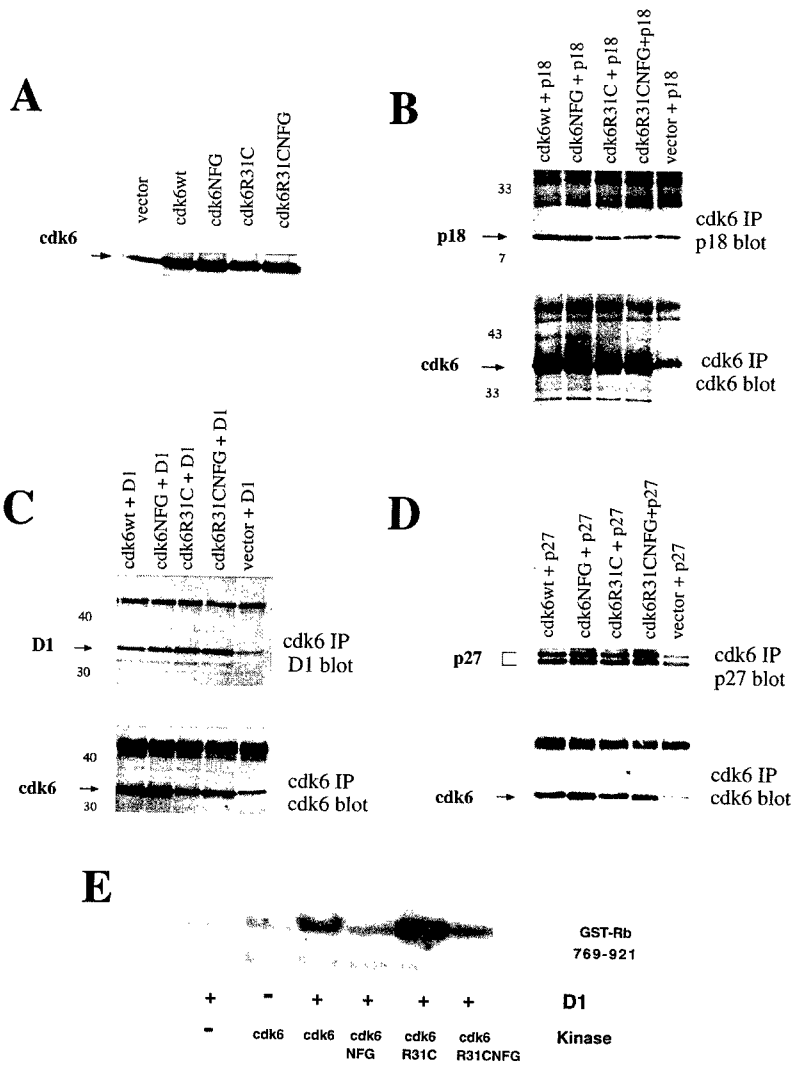


FIG. 3. **Biochemical characterization of cdk6 and cdk6 mutants.** *A*, direct immunoblot of 20 μg of transfected U2OS cell lysate using polyclonal anti-cdk6 antibody C-21. *B*, cdk6 immunoprecipitation of 400 μg of transfected cell lysate immunoblotted with polyclonal p18 antibody and then reprobed with cdk6 antibody, as noted. Anti-p18 immunoblots of these lysates demonstrated equivalent levels of p18 expression in each case (not shown). *C*, cdk6 immunoprecipitation of 200 μg of U2OS lysate immunoblotted with monoclonal cyclin D1 antibody and cdk6 antibody, as noted. *D*, cdk6 immunoprecipitation of 400 μg of cell lysate immunoblotted with polyclonal p27 antibody and reprobed with cdk6 antibody, as noted. In *C* and *D*, control immunoblots showed that these lysates contained equivalent levels of cyclin D1 or p27, respectively (data not shown). *E, in vitro* kinase assay. SAOS-2 cells were transfected with cyclin D1 (*lanes 1* and *3–7*) or its empty vector (*lane 2*) and the indicated kinase or its vector. Lysates from each transfection were tested for their kinase activity using C-terminal GST-Rb substrate (amino acids 769–921).

in S phase population caused by cdk6wt because cdk6R31C, which has demonstrated *in vitro* kinase activity, cannot accelerate G_1 phase. Furthermore, the R31C mutation in cdk6R31CNFG nullifies the S phase inhibitory effect seen after introduction of cdk6NFG, suggesting a critical role for the R31 residue in cdk6 function.

Cell Cycle Effects Correlate with Nuclear Localization—Previous studies have shown that the subcellular localization of cdk4 may influence its interaction with the CIP and INK families of inhibitors (27). In addition, both cdk4 and cdk6 have been observed to localize to the cytoplasm in a variety of cell types (37–39). Thus, we wished to test the hypothesis that the inability of the cdk6 mutants to accelerate G_1 phase may be in part due to differential localization within the cell. Transfected U2OS cells were synchronized using nocodazole and analyzed by indirect immunofluorescence for cdk6. Repeatedly, the mutant forms of cdk6 that failed to bind INK4 proteins (cdk6R31C and cdk6R31CNFG) demonstrated greatly decreased nuclear staining as compared with cdk6wt and cdk6NFG at 8–10 h after mitotic shake (Fig. 5). These results were repeated in at least three separate transfections and with two distinct staining methods using both polyclonal and monoclonal antibodies (Fig. 5).

The decrease in nuclear staining observed in cdk6R31C and cdk6R31CNFG transfectants was also observed in asynchronous populations at 24 h after removal of DNA precipitates, but in these populations the percentage of cdk6R31C and cdk6R31CNFG mutants with predominantly cytoplasmic staining was lower than the percentage seen in a synchronous population (shown in Fig. 5), suggesting that the localization of these kinases is cell cycle-regulated.

Together, these studies demonstrate that the R31C mutation affects compartmentalization of cdk6, as well as the ability to interact with INK4 proteins. R31C mutants showed a remarkable decrease in nuclear staining particularly at time points predicted to be at or near the G_1/S boundary. This difference in compartmentalization directly correlated with the inability of the same mutants to accelerate G_1 phase of the cell cycle and suggested a role for INK4 protein binding domain in the generation of functional, nuclear cdk6 complexes.

DISCUSSION

The D-cyclin-dependent kinases cdk4 and cdk6 share pRb as their only proven physiological substrate, and both can act as oncogenes in human tumors that retain pRb. Previous experiments have identified numerous, tumor-derived cdk4 mutants that fail to interact with $p16^{INK4a}$, suggesting that this kinase acts as an oncogene by evading inhibition by INK4 proteins. This may in turn result in direct modification of substrates by high levels of kinase activity or may produce an indirect effect through increased p21/p27 titration. The results presented here demonstrate that cdk6 can accelerate G_1 phase transit when ectopically expressed in U2OS cells even though these cells do not express $p16^{INK4a}$.

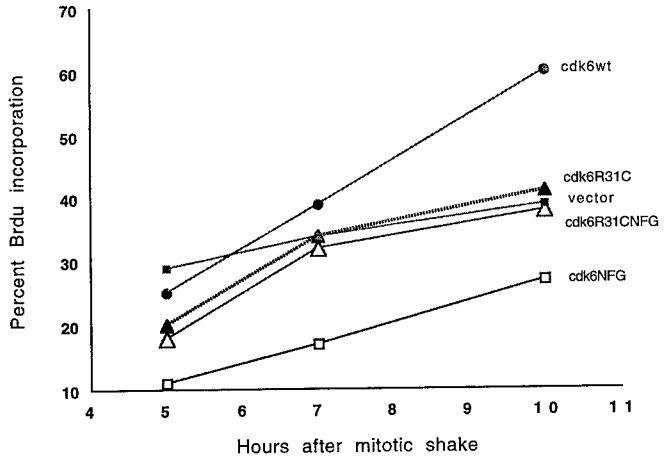


FIG. 4. **BrdUrd incorporation of U2OS cells expressing cdk6 mutants.** Graph of BrdUrd incorporation of kinase-positive cells as determined by immunofluorescence. BrdUrd incorporation of transfected U2OS cells harvested at 5, 7, or 10 h after mitotic shake. At least 120 vector (*closed squares*), cdk6wt (*closed circles*), cdk6R31C (*closed triangles*), cdk6R31CNFG (*open triangles*), and cdk6NFG (*open squares*), transfected cells were scored as BrdUrd-positive or BrdUrd-negative at each time point.

Expression of cdk6 resulted in increased S phase as measured by FACS and BrdUrd incorporation. Nocodazole and mitotic release studies indicated that the observed increase in S phase cells resulted at least in part from decreased transit time through G_1 phase of the cell cycle. Although we cannot rule out an accompanying lengthening of S phase, nocodazole-treated, cdk6-transfected cells did accumulate in M phase, suggesting that any effect of S phase transit time was not large. Surprisingly, the accelerated G_1 phase did not require co-transfection of the kinase-activating partner, cyclin D. G_1 acceleration by cdk6 in the absence of increased cyclin D is consistent with a model in which the supply of cyclin D is not the only rate-limiting step in kinase activation. Indeed, cyclin D1 levels are quite stable across the cell cycle in many proliferating cells, unlike cyclins A, E, and B. Thus, whereas cyclin D complex formation is obviously a critical step in kinase activation, it may not be the rate-limiting step in cultures of proliferating cells.

The availability of the kinase may be particularly limiting for cyclin D/cdk6 complexes, because the INK4 family of inhibitors act as competitors with cyclin D for cdk6 binding. In light of this, the ability of cdk6 to accelerate G_1 in U2OS cells is somewhat surprising, given that U2OS cells lack $p16^{INK4a}$ and thus are thought to be able to phosphorylate and inactivate pRb without hindrance of cdk4 or cdk6 activity. Nevertheless, our results suggest that cdk6 is limiting for cell cycle progression despite the absence of $p16^{INK4a}$. This is consistent with the observed ability of excess cdk4 to increase the proliferation of astrocyte cell lines lacking $p16^{INK4a}$ (40). One possibility arising from such observations is that cdk6 can act "noncatalytically" in this system by sequestering p21 or p27 away from cdk2. Such a role for cyclin D/cdk4(6) complexes is strongly supported by experiments demonstrating that $p15^{INK4b}$ and $p16^{INK4a}$ can cause redistribution of p21 and p27 from cyclin cdk4(6) complexes to those containing cdk2, thereby augmenting cell cycle arrest (26, 27, 41, 42).

The role of cyclin D/cdk4(6) complexes as "sinks" for p21 and p27 would suggest that catalytic activity of cdk6 would be dispensable for G_1 acceleration if titration alone were sufficient to shorten G_1. Our results using cdk6NFG, which is catalytically inactive yet still able to bind D cyclins and p27, argue that such p21 and p27 titration is not responsible for cdk6-mediated G_1 acceleration in U2OS cells. cdk6NFG was completely incapable of shortening G_1 phase in these experiments, and indeed it detectably delayed S phase entry as determined by BrdUrd incorporation following release from nocodazole (Fig. 4). These results strongly argue that catalytic activity of cdk6 is required for the observed effects on cell cycle progression in U2OS cells and inhibitor sequestration is not sufficient for this effect.

It is possible that kinase inhibition by other members of the INK4 family limit proliferation in cultured cells lacking $p16^{INK4a}$. For example, G_1 length may be partly determined by the ratio of endogenous cdk6 to $p18^{INK4c}$ (43), which is expressed in U2OS cells, and excess cdk6 would thus result in a shortened G_1 phase. This model predicts that elimination of INK4c binding by the R31C mutation, which is analogous to the oncogenic R24C mutation in cdk4, would enhance the G_1 acceleration function of cdk6. However, our results using cdk6R31C stand in direct contradiction to this, because R31C is completely unable to alter G_1 phase in transfected U2OS cells. Because this mutation, like the NFG mutation, does not disturb properties of cdk6 such as cyclin D1 or p27 binding, this result further argues against a role for inhibitor titration in the G_1 acceleration function of cdk6. Indeed, because cdk6R31C can be activated by cyclin D1 in cotransfected cells, these results suggest that the N terminus of cdk6 may be involved in the proper function of cdk6 within cells (but not *in vitro*), perhaps at the level of substrate recognition or compartmentalization.

Published reports indicate that both cdk4 and cdk6 are indeed regulated at the level of subcellular localization (13, 27, 38). Consistent with these studies, data shown here demonstrate that cdk6 localizes to both the nucleus and the cytoplasm, whereas cdk6R31C preferentially localizes to the cytoplasm. Synchronized U2OS cells show a striking lack of cdk6R31C and cdk6R31CNFG protein in the nucleus in late G_1 phase. Recently, it has been shown that cytoplasmic cdk6 exists primarily in inactive complexes with cdc37 and hsp90 or (in T cells) with $p19^{INK4d}$ (37, 39). In light of this, the localization pattern of the cdk6R31C protein presents an apparent paradox. Why is an INK4 binding-defective cdk6 protein preferentially localized in the cytoplasm if the major function of the cytoplasmically localized INK4 protein (in this case, specifically $p18^{INK4c}$) is to anchor kinases in an inactive state? We suggest that the N terminus of cdk6 is critically involved in the dissolution of cytoplasmic complexes and may be a binding site for proteins that serve to promote translocation of cdk6 to the nucleus. It is possible that such proteins resemble INK4 proteins, or it may even be the case that INK4 proteins themselves could promote nuclear entry of cdk6 under certain circumstances, because INK4 proteins can compete with cdc37/hsp90 for binding to cdk4 and cdk6 (44).[2] The precise role of the cdk6 N terminus in subcellular localization is currently under investigation, but whatever the mechanism, maintenance of the cdk6R31C mutant in the cdc37 complex predicts a persistence of cytoplasmic localization and a functionally inactive kinase. In fact, this is precisely the phenotype observed with cdk6R31C: an increased cytoplasmic retention (Fig. 5) and a loss of function in either G_1 acceleration (cdk6wt) or G_1 retardation (cdk6NFG) (Fig. 4), despite greater than wild-type catalytic activity of cdk6R31C in *in vitro* kinase assays (Fig. 3).

The requirement for cdk6 nuclear localization in G_1 acceleration, its likely regulation in the cell cycle, and the novel role of the N terminus in this localization raise the possibility of the existence of a cdk6 regulatory pathway that may result in differential activity of cdk4 and cdk6 in the same cell. Indeed,

[2] M. Grossel and P. Hinds, unpublished observations.

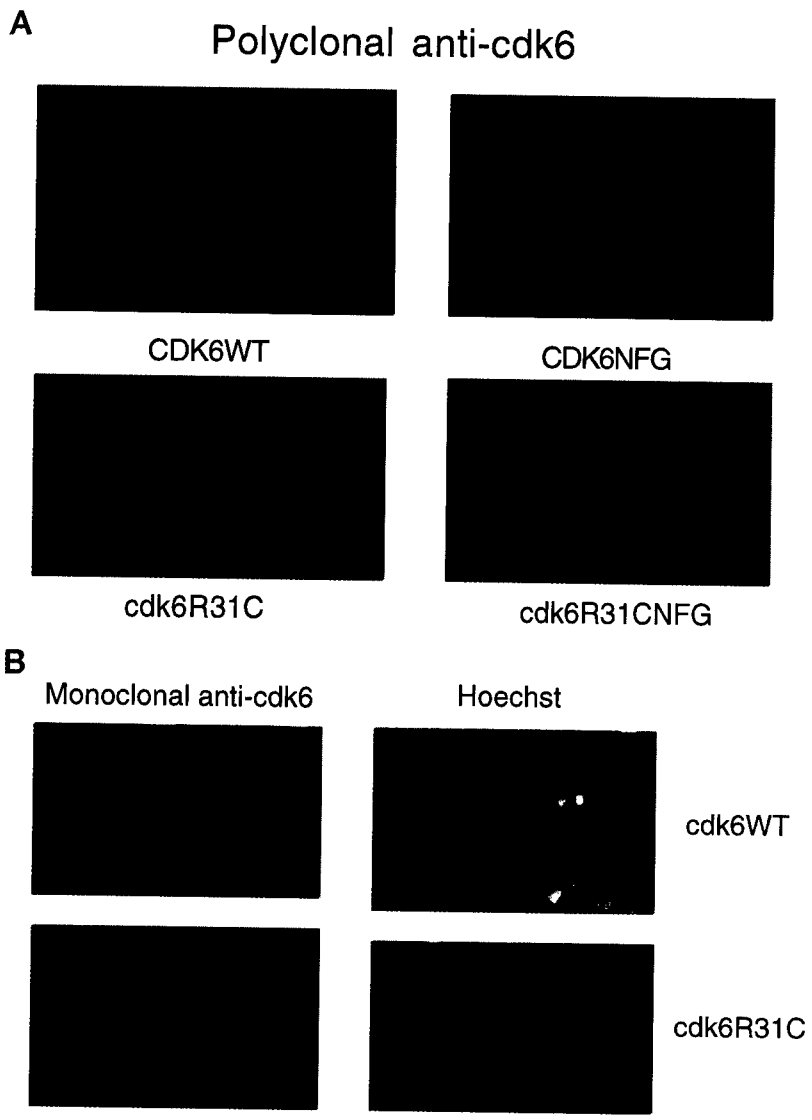


FIG. 5. **Localization of kinases.** Indirect immunofluorescence of transfected cdk6 or mutant forms of cdk6. *A*, cells were fixed at 10 h after synchronization and stained with polyclonal antibody to cdk6 (C-21). *B*, cells were fixed at 9 h after synchronization and stained with monoclonal antibody to cdk6 (Ab-3).

the fact that cdk4R24C has been reported to be hyperactive but cdk6R31C is inactive in cell cycle progression suggests that these kinases may be subject to discrete activation programs. In the case of cdk6, production of active complexes may require the presence of a factor that interacts with the N terminus and that is itself subject to cell cycle regulation. If this factor is not required by cdk4, the two kinases could respond differently to extracellular signals, and this in turn could favor activation of one kinase *versus* another in tumor cells. Clearly, a better understanding of the role of the cdk6 N terminus in functional regulation and the identification of putative activating factors that interact with this domain are required to fully understand the role of cdk6 in cancer cells.

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